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22 APR 1998

1. Your reference

P21483/CPA/RMC

2. Patent application number

*(The Patent Office will fill in this part)***9808407.2**

22 APR 1998

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*The Queen's University of Belfast
School of Biology and Biochemistry
BELFAST
BT9 7BL
Northern IrelandPatents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

UK

7422330001

4. Title of the invention

"Medicament"

5. Name of your agent *(if you have one)*"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*Murgitroyd & Company
373 Scotland Street
GLASGOW
G5 8QA
United KingdomPatents ADP number *(if you know it)*

1198013

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Country

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Number of earlier application

Date of filing
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Description 24

Claim(s)

Abstract

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Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (Patents Form 7/77)

Request for preliminary examination and search (Patents Form 9/77)

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Any other documents (please specify)

11. I/We request the grant of a patent on the basis of this application.
- Signature *Murgitroyd & Company* . Date 21 April 1998
Murgitroyd & Company
12. Name and daytime telephone number of person to contact in the United Kingdom Roisin McNally, 0141 307 8400

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1 **"Medicament"**

2

3 This invention relates to the use of modified peptides
4 for the treatment of angiogenic diseases and inhibition
5 of the secondary spread of cancer, as well as treatment
6 of *Candida spp.* infections.

7

8 Angiogenic diseases are those disorders which are
9 directly caused by, or complicated by the inappropriate
10 growth of new blood vessels. The major angiogenic
11 diseases include the common metastatic solid tissue
12 cancers (breast, gastrointestinal, lung, prostatic,
13 etc), diabetic retinopathy, neovascular glaucoma,
14 rheumatoid arthritis and psoriasis. Angiogenesis is
15 the rate-limiting step in the growth of secondary
16 tumours; inhibition of their neovascularisation is
17 known to stop their growth.

18

19 In this field it is already known that the native
20 ligand of the 67kDa laminin receptor (67LR) is
21 encompassed by the linear sequence of amino acids 925-
22 933 of the laminin b1 chain (numbering refers to the
23 mature murine laminin b1). Synthetic laminin b1₉₂₅₋₉₃₃
24 (single letter amino acid code: CDPGYIGSR-NH₂) has been
25 shown to inhibit tumour establishment in mice, by

1 inhibiting attachment of tumour cells to basement
2 membranes. It has also been demonstrated that laminin
3 bl₉₂₅₋₉₃₃ inhibits angiogenesis in the chick.

4
5 However, synthetic laminin-derived peptide (laminin
6 bl₉₂₅₋₉₃₃) stimulates angiogenic events in mammalian cells
7 (in which it acts as a pure 67LR agonist), making it
8 useless as the basis of a human therapy. This dual
9 action suggests that the effects of EGF on motility are
10 mediated by downstream action of 67LR.

11
12 It is one object of the present invention to provide a
13 medicament to treat angiogenic diseases.

14
15 The present invention provides a peptide factor derived
16 from murine epidermal growth factor (EGF) peptide for
17 use in the preparation of a medicament for the
18 treatment of angiogenic diseases.

19
20 The mechanism by which EGF derived peptides inhibit new
21 blood vessel formation is through their antagonism of
22 the high affinity 67 kDa laminin receptor (67LR) found
23 on endothelial cells.

24
25 The peptides have the additional effect of inhibiting
26 tumour cell attachment to basement membranes, and may
27 be used to prevent solid cancer spread in cases where
28 cancer cells have been identified circulating in the
29 blood.

30
31 The modified peptides are protected from proteolytic
32 degradation by substitution of key residues with
33 unnatural amino acid analogues at susceptible bonds,
34 such as tyrosine analogues (at position 5) and arginine
35 analogues (at position 9). The peptides are capped at
36 N- and C-termini (with acetyl and amide groups

1 respectively) and at the thiol groups of the cysteines
2 (with acetamido methyl groups).

3

4 Typically the peptide is an antagonist of the 67kDa
5 Laminin Receptor (67LR).

6

7 The peptide factor is based on amino acid residues 32
8 to 42 of murine epidermal growth factor (mEGF).

9

10 The amino acid sequence of mEGF- (33-42) is CVIGYSGDRC.

11

12 Preferably the sequence of peptide factor is modified
13 from the natural sequence to protect the peptides from
14 protease attack.

15

16 Preferred substitutions include the use of tyrosine
17 analogues at position 5 and arginine analogues at
18 position 9.

19

20 Preferably the peptide factor is capped at the N
21 terminal with an acetyl group.

22

23 Preferably the peptide factor is capped at the C
24 terminal with an amide group.

25

26 Preferably the thiol groups of cysteines are capped
27 with acetamido methyl groups.

28

29 In one embodiment the synthetic peptide has the
30 sequence

31

32 Acetyl-C-[S-Acm]-VIGYSGDR-C-[S-Acm]NH₂

33

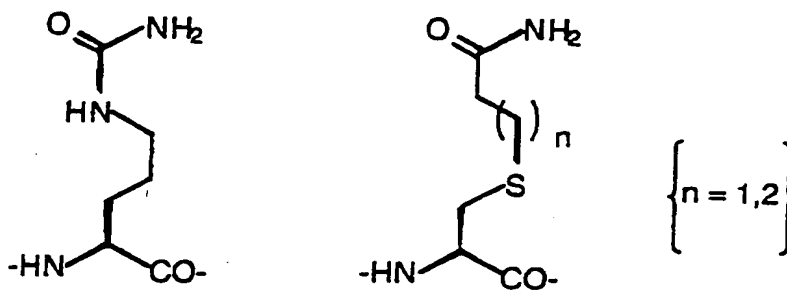
34 A preferred tyrosine analogue is Tic-OH.

35

36 A preferred arginine analogue is Citrulline.

The structure of Citrulline and other potential arginine analogues are shown below.

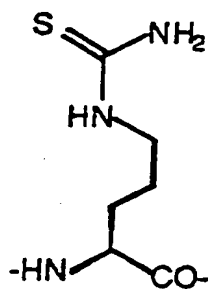
Citrulline and analogues



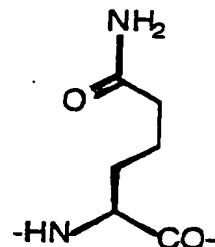
citrulline

cysteine-derived analogues

{prepared by reaction of cysteine with $\text{Br}-(\text{CH}_2)_n-\text{CONH}_2$ }



thiono-citrulline



homo-glutamine

{prepared by reaction of ornithine with ammonium isothiocyanate}

Preferably the peptide is truncated to a shorter peptide without losing its antagonistic character.

The invention further provides a peptide agonist.

The agonist (typically containing Tic-OH substitution for tyrosine) might be useful in healing endiothelial

1 cell wounding.

2

3 For example, corneal endothelial cells can be damaged
4 during cataract operations and this damage does not
5 self-repair because these endothelial cells do not
6 divide. Healing can only be by cell migration and
7 spreading, and this may be promoted by the agonist.

8

9 In order to explore possible conformations for the
10 parent mEGF₃₃₋₄₂ peptide, it was modelled using molecular
11 dynamics. Based on these conformations we were able to
12 predict a strategy to provide proteolytic protection by
13 being able to identify residues that were important to
14 the maintenance of a three-dimensional conformation
15 essential for 67LR recognition.

16

17 The following is a description of some examples of
18 modifications and uses of the invention.

19

20 1. On the basis of the modelled structures, we found
21 that the arginine residue participated in H-
22 bonding, and speculated that this charge may not
23 be important. We synthesised a peptide based on
24 mEGF₃₃₋₄₂, in which the arginine residue at position
25 41 was replaced by citrulline (an uncharged
26 arginine mimetic with similar H-bonding
27 potential). This peptide provided to act as a
28 more potent 67LR antagonist and was found to be
29 resistant to trypsin degradation.

30

31 2. Double substitution of tyrosine₃₇ with Tic-OH and
32 Arginine₄₁ with citrulline, to produce a mEGF₃₃₋₄₂-
33 derived peptide resistant to both chymotrypsin-
34 like and trypsin-like proteases.

35

36 3. Replacement of susceptible peptide bonds in mEGF₃₃₋

42 with protease-resistant peptide bond isosteres
(such as thiono peptide or methylene amino bonds).

4. Conformationally restricted analogues may give improved potency due to the essential 3-dimensional conformation being stabilised. For example, it should be possible to increase the rigidity of the molecule by replacing each of the central glycine residues in turn by α,α -dialkyl substituted amino acids such as α -amino isobutyric acid (AIB) or aminocyclopropane carboxylic acid (ACPCA). Alternatively, the helical turn (which we have identified as essential) could be stabilised by bridging with suitable intra-chain linkers, such as a disulphide bond between *N*- and *C*-terminal [*D*] or [*L*]-cysteines.

Alternative Uses

1. Some microbial pathogens such as *Candida albicans*, express 67LR and use this as a means of attaching to human basement membranes. It is conceivable that such infections could be abolished by treatment with mEGF₃₃₋₄₂-derived peptides, which would prevent the microbes from adhering to the host.

Advantages

The advantages of the invention, and the ways in which disadvantages of previously known arrangements are overcome include:

1. Unlike the native 67LR ligand (laminin b1₉₂₅₋₉₃₃), which is angiogenic in human models, the mEGF₃₃₋₄₂-derived agents are anti-angiogenic in human

1 models.

2

3 2. mEGF₃₃₋₄₂ has the advantage of inhibiting both
4 laminin- and EGF-stimulated angiogenesis.

5

6 3. mEGF₃₃₋₄₂ prevents tumour cell attachment to
7 basement membranes.

8

9 Examples

10

11 The purpose of the investigation was to determine the
12 molecular target of mEGF₍₃₃₋₄₂₎ and to identify the amino
13 acids that are essential for receptor recognition. In
14 addition, the key residues which confer laminin
15 antagonism on mEGF₍₃₃₋₄₂₎ were examined.

16

17 Two lead compounds were investigated; synthetic laminin
18 B1 sequence CDPGYIGSR-NH₂ and mEGF₍₃₃₋₄₂₎ sequence
19 AcC(Acm)-VIGYSGDRC-(Acm)-NH₂. Bearing in mind the pure
20 antagonism of the murine EGF peptide, the aims of this
21 study were to identify the key residues responsible for
22 these contrasting activities using alanine scanning, in
23 the context of developing anti-angiogenic drugs for
24 retinopathy treatment.

25

26 In addition, using residue exchange between the two
27 peptides and molecular modelling to predict three-
28 dimensional structure, we wished to further investigate
29 the role of individual mEGF₍₃₃₋₄₂₎ residues in laminin
30 antagonism. A logical series of peptides was
31 synthesised and screened for receptor interaction, cell
32 adhesion and motility properties (Table 1a and 1b).

MATERIALS AND METHODS

Peptide synthesis

Peptide sequences based on and mEGF₍₃₃₋₄₂₎ were synthesised on a model 432A peptide synthesiser (Applied Biosystems, Warrington, UK), using standard solid-phase Fmoc procedure (Fields 1990). Synthesis of the peptides required successive additions of derivatized amino acids to form a linear product.

Peptides were purified after synthesis using reverse phase HPLC and purity confirmed by automated amino acid analysis and electrospray mass spectrometry. All peptide sequences were stored in the presence of desiccant at -20°C until required for biological assay.

Laminin receptor antibody production

a. Preparation of MAPs

The peptide sequence (PTEDWSAQPATEDWSAAPT), corresponding to the COOH-terminal end of the human laminin receptor, was used as the antigen template. Derivation of the peptide, based on a CN-Br cleavage fragment of the cDNA sequence encoding human laminin receptor, has been described elsewhere (Wewer et al 1986). The antigen was synthesised as an octomeric peptide derivative (MAPs) using automated Fmoc procedure (Tam 1988).

mEGF ₍₃₃₋₄₂₎	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys- NH ₂
I	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ile	Gly	Asp	Arg	ACM Cys- NH ₂
II	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Ser	Arg	ACM Cys- NH ₂
III	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ile	Gly	Ser	Arg	ACM Cys- NH ₂
IV	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Cit	ACM Cys-NH ₂
V	acetyl	ACM Cys	Val	Ile	Gly	OH Tic	Ser	Gly	Asp	Arg	ACM Cys- NH ₂

Table 1b: Peptide substitution (alanine scanning)

mEGF ₍₃₃₋₄₂₎	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys-NH ₂
VI	acetyl	ACM Cys	Val	Ala	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys-NH ₂
VII	acetyl	ACM Cys	Ala	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys-NH ₂
VIII	acetyl	Ala	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	ACM Cys-NH ₂
IX	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ser	Gly	Asp	Arg	Ala-NH ₂
X	acetyl	ACM Cys	Val	Ile	Gly	Tyr	Ala	Gly	Asp	Arg	ACM Cys-NH ₂

1 **b. Immunisation schedule**

2

3 A pre-immune test bleed (5ml) was obtained from the
4 marginal ear vein of a male New Zealand White rabbit
5 (3.2 kg). The bleed was allowed to clot for 2 h at room
6 temperature after which its edge was detached from the
7 wall of the collection vessel. The clot was then
8 allowed to contract overnight at 4°C. Serum was then
9 removed and the residual material pelleted out by
10 centrifugation (10 min at 2,500 g). Extracted serum
11 (3.5 ml) was then frozen at -20°C until required.

12

13 Immunogen was prepared by the emulsion of MAPs (0.5 g
14 antigen in 0.5 ml PBS) in an equivalent volume of
15 adjuvant (Alum Imject; Pierce, Chester, UK). The
16 animals immune system was primed by introducing
17 immunogen (50 µg) through subcutaneous injection at
18 different sites on the animals back. The rabbit was
19 boosted by both subcutaneous and intramuscular
20 injection, 21 days after priming, using an increased
21 dose of immunogen (800 µg). Subsequent boosts were
22 performed by intramuscular injection after a further 14
23 days (800 µg immunogen), and thereafter at 21 day
24 intervals. Test bleeds were taken 2 days after each
25 boost and the serum extracted as described above. The
26 animal was boosted and bled a total of three times.

27

28 **c. Enzyme-linked immunoabsorbent assay**

29

30 ELISA was used to determine the specificity of the
31 antibody prepared against the synthetic MAPs peptide
32 and to determine the efficacy of binding with respect
33 to that of the linear precursor.

34

35 Peptides were dissolved in distilled water and diluted
36 to 10 µg/ml in coating buffer. Aliquots (100 µl) of

1 either linear or MAPs peptide were then added to the
2 wells of microtitre plates (Microtest III; Becton
3 Dickinson Ltd., Oxford, UK) and incubated overnight at
4 37°C. The wells were then rinsed with 100 µl wash
5 buffer and air dried. Excess adsorption sites were
6 blocked (1 h incubation at 22°C) by the addition of 10%
7 casein in PBS (0.1 ml/well). Subsequent to the removal
8 of casein solution by aspiration, wells were again
9 rinsed with wash buffer and air dried.

10
11 Antisera or pre-immune sera were then serially diluted
12 in PBS and 100 µl of each incubated in peptide coated
13 wells for 1 h at 37°C. After rinsing (0.1 ml wash
14 buffer), 100 µl per well of 5 µg/ml secondary antibody
15 (horse-radish peroxidase-conjugated goat anti-rabbit
16 IgG; Amersham International, Aylesbury, UK) was added
17 to each well and the plates incubated at 37°C for 1 h.

18
19 Wells were again rinsed with wash buffer and 0.1 ml
20 substrate solution (TMB peroxidase) added to each. The
21 plate was then incubated at 22°C for 30 min and the
22 colour reaction stopped by the addition of 0.5M H₂SO₄
23 (0.1 ml/well). Absorbance was measured at 450 nm on a
24 Titertek Multiscan plate reader.

25 26 d. Purification of IgG fraction

27
28 Anti-laminin receptor antiserum was purified using
29 immobilised protein G-sepharose columns (Pharmacia
30 Biotech, Uppsala, Sweden). The columns were
31 equilibrated with 20 ml sodium phosphate buffer (pH
32 7.0). Antiserum was diluted 1:4 in the same buffer and
33 a 1 ml aliquot loaded onto the column (flow rate 150
34 ml/h, fraction size 2.5 ml). After exclusion of the
35 unbound fraction, as determined by absorbance at 280nm,
36 the IgG component of the antiserum was eluted with 0.1M

glycine-HCl (pH 2.7), into tubes containing 0.1 ml Tris (1M), pH 9.0. The eluted IgG fractions were bulked and stored at -20°C until required.

Maintenance of cell cultures

Cancer and endothelial cells were maintained in either DMEM (T47-D) or RPMI (SK HEP-1) media, supplemented with 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air: 5% CO₂ and media refreshed as required. Cultures (at 80-85% confluence) were routinely passed on removal from monolayer by the action of trypsin (0.25%) and EDTA (0.02%) in CFS.

The viability of cell populations following trypsinisation was determined by the trypan blue vital dye exclusion test. Populations confirmed as being in excess of 95% viable were used in all studies.

Media were screened for possible bacterial or fungal contamination by incubating 1ml aliquots with both nutrient and Saboraud dextrose broths (Oxoid Ltd., Basingstoke, UK). Cell populations were routinely monitored for sub-clinical infections by periodically culturing in the absence of antibiotics.

Both cell lines and media were examined for the presence of contaminating *Mycoplasma* spp. by the method of Chen (1977).

Determination of cell numbers

Single cell suspensions were quantified using an automated counter (Coulter Electronics, Harpenden, UK). A 1 ml aliquot of cell suspension was diluted 1 in 20

1 in Isoton and 0.5 ml samples counted. The mean of 5
2 counts was taken and the total number of cells
3 determined. Estimates of cell number were confirmed by
4 counting in a haemocytometer.

5
6 For microtitre end-point assays, cell numbers were
7 estimated from the crystal violet staining index of the
8 cell line (Kanamaru and Yoshida 1989). Briefly, after
9 removal of media from the assay system cells were fixed
10 with formaldehyde (10% in PBS), and washed with
11 distilled H₂O. Aliquots (100 µl) of crystal violet
12 solution (0.1% in distilled H₂O) were added to each well
13 and the plates allowed to stand for 30 min. Excess
14 stain was removed by rinsing with distilled H₂O (3 x 100
15 µl). The wells were then air-dried and the remaining
16 crystal violet extracted with 100 µl acidified
17 methanol. Absorbance at 620 nm was determined using a
18 Titertek Multiscan spectrophotometer.

19 20 Proliferation assays

21
22 The effects of synthetic peptides and growth factors on
23 the growth of breast cancer and endothelial cells were
24 determined as detailed.

25
26 Exponentially growing cells were harvested by
27 trypsinisation, as previously described. After rinsing
28 and resuspending in the relevant culture media
29 (containing 10% FCS), the cells (100 µl aliquots) were
30 dispensed into 96-well microtitre plates at a
31 population density of 2×10^4 cells/well (6 wells per
32 experimental condition). Cells were incubated for
33 24 h at 37°C after which the media was removed and the
34 wells rinsed with CFS (3 x 100 µl), to rid the plates
35 of cells in suspension. Media was then replaced with
36 that containing the relevant controls or treatment

1 supplements as detailed in individual experiments.

2

3 Cell numbers were evaluated spectrophotometrically at
4 620 nm, over the period of assay, after fixing with 10%
5 formaldehyde and staining with crystal violet.

6

7 Proliferative responses were analysed using the
8 Wilcoxon Rank test and significant differences at the p
9 < 0.05 level, defined. Results of all growth studies
10 were confirmed in at least 3 individual experiments.

11

12 Laminin attachment assay

13

14 Non-tissue culture grade 96-well plates, coated with
15 2.5 μg murine laminin in 50 μl CFS per well, were air-
16 dried overnight at room temperature. Preliminary
17 experiments indicated that cell attachment was
18 concentration dependent; maximal binding occurred at a
19 laminin coating of 2.5 $\mu\text{g}/\text{well}$. After rinsing with CFS
20 (100 μl), the plastic was saturated with casein (0.2%
21 in CFS). Plates were incubated at room temperature for
22 45 min then washed extensively with CFS (3 x 100 μl).

23

24 After removal of culture media, cells were detached
25 from monolayers by the action of EGTA (0.02% in CFS) at
26 37°C. The cells were then centrifuged at 800 g for 2
27 min and the pellet resuspended in DMEM (T-47D) or RPMI
28 (SK HEP-1).

29

30 Cells, at a population density of 10^6 cells/ml, were
31 then aliquoted (1 ml) into microfuge tubes containing
32 the individual peptide sequences and incubated for 1 h
33 at 37°C. The cells (100 μl aliquots) were then added to
34 the pre-coated multi-well plates and incubated for a
35 further 60 min. Incubation media were removed and the
36 wells washed with CFS (3 x 100 μl) to rid the plates of

1 non-adherent cells.

2

3 Attached cell numbers were evaluated
4 spectrophotometrically at 620 nm after fixing with 10%
5 formaldehyde and staining with crystal violet.

6

7 Attachment to mEGF₍₃₃₋₄₂₎

8

9 That mEGF₍₃₃₋₄₂₎ bound to the 67kDa laminin receptor was
10 demonstrated using a biotinylated derivative of the
11 peptide (Acetyl-C-[S-Acm]-VIGYSGDR-C-[S-Acm]-K-[N^F-
12 biotin]-amide) and a modification of the above laminin
13 attachment assay.

14

15 Briefly, 96-well plates were coated with 100 µl/well
16 streptavidin (5 µg/ml in carbonate buffer pH 9.6) and
17 following an overnight incubation at 37°C, wells were
18 washed with CFS (3 x 100 µl) and the plastic blocked
19 with casein (0.2% in CFS). The plates were then
20 incubated at room temperature for 45 min and washed
21 with CFS as previously detailed. Biotinylated mEGF₍₃₃₋₄₂₎
22 in CFS was then aliquoted into the wells (0.1 ml of 100
23 µM) and the plates incubated for 3 h at 37°C.

24

25 After a further block with 0.2% casein, the wells were
26 washed with with CFS (3 x 100 µl aliquots). Plates were
27 kept at 4°C and used within 2 h.

28

29 Cells were prepared as above and pre-incubated for 1 h
30 at 37°C with serial dilutions of anti-laminin receptor
31 polyclonal (see below) or anti-EGF (R1) receptor
32 monoclonal antibodies. Subsequent procedures were as
33 detailed for the laminin attachment assay.

34

35

Laminin receptor binding determinations

a. Radiolabelling of laminin

^{125}I -laminin was prepared using ^{125}I -labelled sodium iodide (Amersham, UK) and immobilised chloramine-T (Iodobeads; Pierce, Illinois). Prior to use, the beads were washed with 500 μl phosphate buffer (pH 6.5) to remove excess reagent from the support. These were then allowed to air dry and individual beads added to a solution of carrier free Na^{125}I , diluted with iodination buffer (phosphate buffer pH 7.4). The beads were allowed to equilibrate for 5 min.

Laminin (10 μg in 10 μl) was then diluted into the iodination buffer and the system incubated at 20°C for 15 min. The solution was then removed from the reaction vessel and excess Na^{125}I and unincorporated $^{125}\text{I}_2$ separated from the iodinated protein by gel filtration on a GF-5 exclusion column (Pierce, Illinois). Iodinated laminin fractions were recovered at a specific activity of approximately 1.2 mCi/mg protein (864 Ci/mmol).

b. Competition binding estimation

Near confluent cultures of T47-D or SK HEP-1 cells were removed from monolayer with 0.02% EGTA and passed through a G-25 syringe needle to produce single cell suspensions. Aliquots of each cell type (10^6 cells/ml) were dispensed into separate Ependorf tubes (1 ml each) and pelleted. The cells were then resuspended in 1 ml ice-cold RPMI (SK HEP-1) or DMEM (T47-D) containing 0.1% BSA and either laminin or synthetic peptide at the concentrations indicated. Iodinated laminin was then added to each cell suspension to give a final ^{125}I -

1 laminin concentration of 0.1 nM (approximately 50,000
2 cpm). These mixtures were incubated overnight at 4°C.

3
4 The tubes were then microfuged at 10,000 g and the
5 supernatant removed. After washing the pellet with 500
6 µl CFS, the remaining radioactivity was determined
7 using a gamma radiation counter. Non-specific binding
8 was determined by incubating cells with a 1000-fold
9 molar excess of unlabelled laminin. All estimations
10 were carried out in triplicate.

11
12 IC₅₀ (concentration of unlabelled peptide required to
13 produce 50% inhibition of radioligand binding binding)
14 and EC₅₀ (effective concentration for 50% inhibition of
15 cell attachment) values were calculated using the
16 Grafit curve-fitting programme (Erithacus Software,
17 London, UK).

18

19 Migration assays

20

21 The method used was basically as described by Albrecht-
22 Buehler (1977). Briefly, coverslips (22 x 22 mm) were
23 treated in 5% detergent (7X; ICN Biomedicals) and
24 washed in alcohol to remove grease. After drying, they
25 were immersed in gelatin solution (Sigma, 300 Bloom;
26 0.5 g in 300 ml distilled H₂O) for 10 min. The
27 coverslips were then dried by placing in a 70°C oven
28 for 45 min.

29

30 Colloidal gold suspension was prepared by adding 11 ml
31 distilled H₂O and 6 ml Na₂CO₃ (36.5 mM) to 1.8 ml AuHCl₄
32 (14.5 mM). The mixture was heated to 95°C at which
33 point 1.8 ml of freshly prepared 0.1% formaldehyde
34 solution was added; the temperature was maintained at
35 95°C. A suspension of colloidal gold was formed which
36 was brown to absorbed light and blue to transmitted

1 light.

2

3 The gold suspension, was then added to petri dishes
4 containing individual coverslips and the plates
5 incubated at 37°C for 45 min. After washing with CFS (3
6 x 4 ml) to remove unattached gold particles, the
7 coverslips were transferred to 6-well cluster dishes
8 and UV sterilised.

9

10 Endothelial cells (SK HEP-1 and BRCE) in culture media
11 (0.3 ml) were seeded onto the coverslips at an
12 approximate density of 5×10^3 cells per well. The cells
13 were allowed to plate down for 2 h at 37°C after which
14 the treatments were added. Assay systems were
15 maintained for a further 18 h after which the cells
16 were fixed using 3% gluteraldehyde in cacodylate buffer
17 (pH 7.2).

18

19 The assays were examined using a Leica DM1RB phase
20 contrast microscope and Q500MC image analysis system
21 incorporating a JVC TK-1280E colour camera (Leica,
22 Milton Keynes, UK). The track images of at least 30
23 cells were video-captured and the area (representing
24 migration response) determined for each. Statistical
25 analysis of these areas was then carried out using
26 Macintosh Instat software to perform both Kruskal-
27 Wallis analysis of variance and Mann-Whitney *U*-tests in
28 order to compare the treatment groups with controls.

29

30 RESULTS

31

32 Proliferative response

33

34 All peptides were examined for their ability to
35 influence the growth of T47-D and SK-Hep 1 cell lines.
36 At concentrations of peptide up to 100µM, no

1 significant effects were observed in either cell line.

2

3 **Mechanism of action**

4

5 We had shown in earlier studies that mEGF₍₃₃₋₄₂₎ could
6 inhibit the EGF-stimulated angiogenic response in the
7 early chick as well as blocking the basal and EGF-
8 stimulated motility of primary and established
9 endothelial cells.

10

11 During the present study we have shown that mEGF₍₃₃₋₄₂₎
12 also inhibits the angiogenic effects of laminin (Nelson
13 et al 1995). Furthermore, we have demonstrated that the
14 anti-angiogenic effects of mEGF₍₃₃₋₄₂₎ are mediated solely
15 through the high affinity 67 kDa laminin receptor (67-
16 LR) and not through the EGF receptor.

17

18 We have also confirmed that mEGF₍₃₃₋₄₂₎, Lam.B1₍₉₂₅₋₉₃₃₎ and
19 laminin are equipotent in ¹²⁵I-laminin displacement
20 receptor assays, and that both of the small peptidal
21 ligands have similar potencies in specific laminin cell
22 attachment assays.

23

24 In addition, we have shown that the commonly used chick
25 angiogenesis models are not appropriate to the study of
26 laminin mediated human angiogenesis: although we
27 confirmed that Lam.B1₍₉₂₅₋₉₃₃₎ acts as a partial laminin
28 antagonist in chick, we found it to be a pure agonist
29 in mammalian cell lines. This is a highly significant
30 point given that pharmaceutical companies (such as
31 Angiotech, Vancouver, BC) are using the chick CAM assay
32 as the sole screening method for the discovery of anti-
33 angiogenic lead compounds. This may be inappropriate
34 for use in human disease.

35

36 We were the first to show that the YIGSR-receptor is,

1 in fact, the 67 kda high affinity laminin receptor (67-
2 LR). In collaboration with Professor Archer's team at
3 the Department of Ophthalmology, Royal Victoria
4 Hospital, Belfast, we have now determined that the 67-
5 LR is preferentially expressed in new vessels during
6 oxygen-induced retinopathy in neonatal mice (Nelson et
7 al 1997a; manuscript in preparation).

8 9 Peptide antagonist development

10
11 The N-terminus of Lam.B1₍₉₂₅₋₉₃₃₎ is not necessary for
12 receptor recognition and the agonist activity of YIGSR
13 peptide (Ostheimer et al 1992, Kawasaki et al 1994).

14
15 However, alanine scanning of the starting peptide
16 (mEGF₍₃₃₋₄₂₎) indicated that residues at positions 1, 2,
17 3, and 6 (peptides VI, VII, VIII and X respectively),
18 are essential for receptor mediated activities as
19 determined by ¹²⁵I-laminin displacement and cell
20 attachment to laminin through the 67-LR. Substitution
21 of these individual residues by alanine leads to a
22 dramatic decrease in receptor affinity observed as an
23 increased IC₅₀ (Table 2) and a parallel decrease in
24 their ability to block adhesion to laminin (increased
25 EC₅₀; Table 2). Characterisation of these analogues with
26 regard to effects on motility, largely confirmed these
27 findings although there was one exception; peptide
28 VIII. Results from the migration assay identified this
29 sequence (alanine for cysteine (P1)) as being a weak
30 laminin agonist despite there being a much reduced
31 response in the other two assays. It is suggested that
32 this peptide may influence laminin receptor mediated
33 migration through an alternative mechanism (Scott
34 1997).

35
36 Substitution at P10 (alanine for cysteine (peptide X))

1 retains both receptor binding and adhesion displacing
2 activities but has the effect of changing the
3 antagonistic parent into an agonist analogue. This
4 reflects the response the agonism of Lam.B1₍₉₂₅₋₉₃₃₎, which
5 also lacks the C-terminal cysteine, and suggests that
6 this cysteine is not essential for receptor
7 recognition, but is required for antagonism of mEGF₍₃₃₋₄₂₎.

10 Studies have reported that the positive charge offered
11 by arginine (P9) is essential for the biological
12 activity of Lam.B1₍₉₂₅₋₉₃₃₎ (McKelvey et al 1991, Kawasaki
13 et al 1994). Glutamate substitution for arginine
14 generates a negative charge at this position with
15 corresponding loss of biological activities (Kawasaki
16 et al 1994).

18 However, the substitution of arginine (P9) with
19 positively-charged lysine (McKelvey et al 1991) also
20 results in complete loss of ligand binding and
21 biological activities, suggesting that the mere
22 presence of a positive charge at this position is, in
23 itself, insufficient for receptor recognition. Our
24 modelling studies suggest that H-bonding of the
25 guanidino group of the arginyl residue to the aromatic
26 sidechain of the tyrosyl residue (P5) in the consensus
27 sequence GYXGXR presents an acceptable motif for 67-LR
28 activation by both mEGF₍₃₃₋₄₂₎ and Lam.B1₍₉₂₅₋₉₃₃₎.

30 Substitution of tyrosine (P5) with a conformationally
31 restricted mimetic (tetrahydroisoquinoline-3-carboxylic
32 acid; Tic-OH) in peptide V converted the antagonist
33 mEGF₍₃₃₋₄₂₎ into an agonist. This residue substitution
34 generates a predicted conformation unlikely to be able
35 to form H-bonds. Although both receptor binding and
36 adhesion responses were retained in this peptide the

1 loss of antagonism would suggest that H-bonding between
2 tyrosine (P5) and the arginine (P9) is important for
3 these antagonist activities.

4
5 Modelling studies suggested that citrulline (an
6 uncharged arginine mimetic) would also be capable of
7 forming this H-bonded motif.

8
9 It was found that replacement of arginine (P9) with
10 citrulline (peptide IV) increased both receptor binding
11 and inhibition of attachment to laminin substrata
12 whilst retaining antagonist migratory response (Table
13 2), reinforcing our observation that it is not the
14 positive charge that is required rather than a active
15 conformation generated by hydrogen bonding. These
16 findings thus identify H-bonding between P5 and P9 as
17 being more important than the charge at the P9 arginine
18 in determining antagonist activity.

19
20 Subsequent strategies involved the substitution of
21 variant residues in the antagonistic mEGF₍₃₃₋₄₂₎ with
22 those present in the agonistic Lam.B1₍₉₂₅₋₉₃₃₎ sequence
23 (peptides I-III), in an effort to identify key amino
24 acids in the C-terminal regions (P5-10) of the two
25 ligands responsible for their contrasting
26 bioactivities.

27
28 Substitution of isoleucine (P6) for serine (peptide I)
29 resulted in both reduced receptor affinity and potency
30 in displacement of cell adhesion to laminin.
31 However, this analogue retained weak antagonist
32 activities in the motility assay. It is therefore of
33 interest that studies on the YIGSR sequence indicate
34 that residue substitution, at the position taken by
35 isoleucine in the pentapeptide, are well tolerated and
36 may increase potency (Kawasaki et al 1994).

1 Replacement of aspartate (P8) with serine (peptide II)
2 resulted in a complete loss of biological function. as
3 did peptide III encompassing both the former
4 (isoleucine (P6) for serine) and latter (serine (P8)
5 for aspartate) substitutions. Since this mEGF₍₃₃₋₄₂₎
6 analogue sequence (peptide II) encompasses the active
7 YIGSR amino acid sequence agonist, it is suggested that
8 this loss of activity may be attributed to the valine
9 (P2) and isoleucine (P3) residues in the *N*-terminal
10 half of mEGF₍₃₃₋₄₂₎. Alternatively, addition of a C-
11 terminal cysteine to the YIGSR sequence is known to
12 reduce potency (Kawasaki et al 1994). Additional
13 peptides incorporating the valine (P2) and isoleucine
14 (P3) substitutions are currently under investigation.
15

16 The determination of the minimum core peptide structure
17 is ongoing and involves similar characterisation
18 studies on a number of sequences truncated at the C-
19 terminal.
20

21 These studies have thus identified an important
22 antagonist of 67-LR mediated activities in peptide IV.
23 The sequence, (AcC(Acm)-VIGYSGD-[Cit]-C-(Acm)-NH₂.), may
24 provide an important template for anti-angiogenic drugs
25 in that it is resistant to cleavage by trypsin-like
26 proteases and has been identified as being more potent
27 than mEGF₍₃₃₋₄₂₎ in our screening procedures.
28
29
30